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Original Research

Cell dedifferentiation, callus induction and somatic embryogenesis in *Crataegus spp*

N. Taimori[1], D. Kahrizi[2, 3 *], V. Abdossi[1], A. H. Papzan[4]

[1] Department of Horticulture, Islamic Azad University, Science and Research Branch, Tehran, Iran
[2] Biology Research Center, Kermanshah University of Medical Sciences, Kermanshah, Iran
[3] Department of Agronomy of and Plant Breeding, Faculty of Agriculture, Razi University, Kermanshah, Iran
[4] Department of Agricultural Extension and Education, Razi University, Kermanshah, Iran

Abstract: The present study describes the effects of light conditions, different kinds and concentrations of auxins [Naphthylacetic acid (NAA) and dichlorophenoxyacetic acid (2,4-D)] with cytokinin (Kin) in MS medium on callus induction and embryogenesis in *Crataegus pseudoheterophylla, C. aronia and C.meyeri.* At first leave explants sections were cultured on different combinations of plant growth regulators in dark and light for callus initiation and light conditions to evaluation the percentage and duration of survival, callus diameter, callus fresh weight and dry. Results of effects of plant growth regulators and light conditions on callus initiation revealed that highest percentage of callus initiation leaves in treatment (0.5 mg/l 2.4-D+0.5 mg/l KIN) for species *C.pseudoheterophylla* in dark conditions (100%). Dark conditions (100%) were more effective on callogenesis than light conditions (Photoperiodicity of 16-h and at light intensity of 40 µmol m^{-2} s^{-1}). The callus induction of *in vitro* (64-100%) leaves was better than the *ex vitro* ones (0-100%). The combination of 2,4-D and Kin of *in vitro* leaves callogenesis has been indicated faster (one weeks) than the other combinations. The results also showed that the highest percentage (100%) and survival duration (6 months) was found in species *C. pseudoheterophylla* and *C. meyeri* in 0.1 mg/l 2,4.D + 0.5 mg/l KIN and 0.5 mg/l 2,4.D + 0.5 mg/l Kin. The minimum survival (0%) was absorbed in species *C. aronia* in 1 mg/l NAA. Maximum callus (10.63 and 10.00 mm respectively) was shown in 0.1 mg/l 2,4.D + 0.5 mg/l Kin and 0.5 mg/l 2,4.D + 0.5 mg/l Kin and was not significant differences after five week among species. The results showed that the highest fresh (1081.49 mg) and dry weight (506.88 and 506.98 mg respectively) was absorbed in species *C. pseudoheterophylla* in 0.1 mg/l 2,4.D + 0.5 mg/l Kin and 0.5 mg/l 2,4.D + 0.5 mg/l Kin. The embryogenesis was not occurred in any plant growth regulator combinations and species. The results of this study suggested that using 2,4-D with cytokinin (Kin) would be more beneficial for callogenesis.

Key words: Hawthorn, callus induction, embryogenesis, *Crataegus*.

Introduction

Hawthorn (*Crataegus spp*) is a common name of all plant species in the genus *Crataegus* that belongs to the Rosaceae family. In this genus, there are around 280 species (1, 2). This genus contains 22 species and five families in Iran (3). Generally it is distributed in the Northern hemisphere. Hawthorn plants are highly drought tolerance and are recommended for water conserving horticultural practices. In Turkey, wild-grown hawthorn plants are often top worked with pears and less frequently with apples (5). Hawthorns are generally propagated by seed but germination is difficult (6). The hawthorn commonly is valuable grafting-stock for quince and shadbush (7). The hawthorn during the past decade has been classified as an endangered species. In addition hawthorn species are listed as medicinal plants in pharmacopoeias due to their high contents of flavonoides (8). Moreover, many independent studies have been shown that *Crataegus* extracts display strong antioxidant potential (9,10). The studies showed that the extracts reduce blood pressure and total plasma cholesterol (1,11,12). Usually in many cases, the first step in plant tissue culture is callus induction optimization. Callus is an amorphous tissue of proliferating and undifferentiated parenchyma cells which frequently occurs in response to wounding at the cut edge of living tissue. All types of plant organs like roots, stems, leaves, etc. and tissues can be used to induce the growth of callus (13).

The level of plant growth regulators plays a key factor that affects callus formation and growth in the culture medium. The auxin commonly used for callus induction is 2,4-D but NAA and IAA are also used (14). Application of BA and NAA has improved callus induction rate in *Eustomagrandi florum* L. (15). The type and concentration of growth regulators depends strongly on the genotype and endogenous hormone content of explants (13). After being induced, callus tissue can be usually sub-cultured to a new fresh media for further growth. When sub -cultured regularly, callus will exhibit S-shape or sigmoidal pattern of growth during each passage. For many species subculture period ranges from three to six weeks (13). The callus culture in Hawthorn has been reported for the first time in 1977 by Schrall and Becker. The *Crataegus monogyna* callus and cell suspension cultures initiated from floral buds were used for the analysis of polyphenols constituents, their production levels and their antioxidant activity (1,16). Type of medium used for micropropagation is also effective successfully. In the case of ornamental plants which are cultivated *in vitro*, the manipulation of physical culture conditions can drastically ameliorate the final effect of propagation protocols (17,18,19).Somatic embryogene-

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*** Corresponding author:** Danial Kahrizi, Associate Prof. in Department of Agronomy and Plant Breeding, Razi University, Kermanshah, Iran. Email: dkahrizi@yahoo.com

sis through callus culture or leaf of differentiated cells, hypocotyl, zygote embryos, etc. can be achieved under *in vitro* conditions (20,21). Somatic embryogenesis is physiological process that vegetative cells, embryonic cells are induced under conditions representative (22,23). The somatic embryogenesis is involves the development of bipolar embryos from vegetative cells susceptible embryogenic in in-vitro conditions (24). Nugent and Chandler (2001) (25) reported somatic embryogenesis and regeneration by hormones IBA and 2,4-D on the species *Eucalyptus globules*.

The highest frequency of embryogenesis in apricot cultivar Nonko was obtained from immature cotyledons harvested about 80 days after flowering on WPM containing 3% sorbitol,1µm 2,4-D and 1 µm BA after 60 days of culture in the dark at 25°C. Production of somatic embryos in grapes affected by various factors such as genotype (26,27(, the explants type, time of collection (28,29) and culture medium (30). There are some reports that have shown propagated of hawthorn through cutting and seed germination is difficult. Therefore it seems necessary to propagation through tissue culture. Nowadays the important goal of experiments is to elaborate innovative and at the same time economically justified technologies. So far as we know, till now there are not enough reports on the effect of different concentration of hormone, environmental conditions to callus induction and plant regeneration in respect of physiological and morphological development in Hawthorn.

In plant tissue culture auxins are broadly used for callus induction. Auxins are involved in cell division, cell elongation, vascular tissue differentiation, cell dedifferentiation, rhizogenesis and root formation, embryogenesis and inhibition of axillary shoot growth (31,32,33). It seems that auxins cause DNA to become more methylated than usual and this might be necessary for their programming of differentiated cells and make them beginning division (32). The cytokinins (CKs) are derivatives of Adenine and seem to be required to regulate the synthesis of proteins which are involved in the formation and function of mitotic spindle apparatus (31,32). Generally, CKs used in plant tissue culture are BAP, zeatin, kinetin and CK-like TDZ. In sweet potato TDZ or BAP was not recommended for shoot development. However, they were useful for callus induction (34). Many researchers mentioned light had significant effect on callus growth and morphogenesis. The inhibition of axillary shoot proliferation and induction of specific enzyme activity are concerned with the formation of some flavonoid glycoside secondary products (32). In this study the callus induction and embryogenesis of different species of Hawthorn under different hormonal conditions and the compositions was evaluated.

Materials and Methods

Leaves of *Crataegus* species grown under *in vivo* and *in vitro* conditions were used as a source of plant material. The leaves were taken during August from 30 years old tree of *Crataegus* species grown in the free conditions in the Sahneh, Kermanshah, Iran (N34°29ˊE47°42ˊ) and leaves cultured under *in vitro* conditions in Tissue Culture Laboratory of Zagros Bioidea Co., Razi University Incubator, Kermanshah, Iran.

Sterilization of leaves

The explants [average in size 5×5 mm] were placed under running tap water for 30 minutes. Surface sterilization of the samples was conducted using 70% ethanol for 30 seconds followed by being washed for 10 minutes with 1% sodium hypochlorite (NaOcl). The explants were then rinsed with sterile distilled water three times for 15 minutes (about *in vivo* leaves) and were used for induction of callus.

Establishment of callus culture

The *in vitro* explants derived cultured on MS medium (supplemented by 30 g/l sucrose) and in petri (100 ml) containing 25 ml of medium MS with the petri being capped with aluminum foil caps. Agar (8 g/l) was added to the medium after pH being adjusted to 5.7.the petri and medium was autoclaved at 0.12MP a pressure for 20 minutes and at 121 °C. The cultures were grown at 24±1°C in a Photoperiodicity of 16-h and at light intensity of 40 µmol m^{-2} s^{-1} supplied through white fluorescent tubes and dark conditions. The calli formed (1 g) sub-cultured to a new fresh medium at every 5 weeks intervals. The sub-culturing was performed within 5 cycles in order to select for a fast-growing callus line. The frequency of callus induction was calculated according to the following equation:

Callus induction frequency (%) = (number of calli producing explants/total number of explants in the culture) ×100.

Effect of different concentrations of hormone on callus induction of *Crataegus* species

The concentrations of hormones [1, 2, 3 mg/l NAA and 1, 2, 3 mg/l NAA + 0.5 mg/l KIN and 0.1, 0.5 mg/l 2,4-D + 0.5 mg/l KIN] were used for callus induction experiment. *In vitro* and in vivo leaves cultivars of *Crataegus* were placed on MS medium supplemented by above various hormones. The cultured leaves were incubated at 24±1 °C in 16/8 h light/dark cycle and dark conditions for callus induction. Two weeks after culture, the frequency of callus induction (%) and size of callus [(width + length)/2] were recorded.

Effects of different hormone concentrations and species on callus growth

To find out for the best concentration of hormone and species for callogenesis, after calli cultivation, the samples were collected at certain time intervals and growth was determined.

The growth of calli was determined by measuring the increase in fresh weight and dry weight (DW). For dry weight calculation, the calli were placed on a petri dish and dried at 50 °C in an oven to a constant weight. Results were expressed as DW (mg).

Fresh weight (%) = [(Initial weight – final weight)/Initial weight] × 100

Total dry weight of calli was measured after dehydration treatment at 50 °C for two days.

Effect of different concentration of hormone and species on percent and term viability of callus

Percent and term viability of callus cultivars haw-

thorn in different hormonal concentrations were measured within 6 months. Calli formed transferred to a new fresh media at every 2 weeks intervals.

Effect different concentration of hormone, species, culture medium on embryogenesis

The applied hormone treatments were called as T1 to T8 as the following:

After one month calli derived [1(T1), 2(T2), 3(T3) mg/l NAA and 1(T4), 2(T5), 3(T6) mg/l NAA + 0.5 mg/l KIN and 0.1 (T7), 0.5 (T8) mg/l 2,4-D + 0.5 mg/l KIN] were transferred to somatic embryogenesis medium. We used MS and 1/2 MS medium for induction of somatic embryos that supplemented by free hormone,1, 2, 3 mg/l TDZ, 5 to 2.5 to 0 mg /l 2,4-D and 2.5 to 0 mg/l 2,4-D for two weeks (each concentration for two weeks). Then media were transferred to MS and 1/2 MS medium (free - hormone) for three months.

Experimental Design and Statistical Analysis

The experiments were conducted using factorial experiment with Completely Randomized Design (CRD) with three replications. The mean comparison test was LSD. Analysis of variance (ANOVA) was performed using SAS computer package and the results has been shown as means ± standard deviation (SD).

Results

Effect of different concentration of hormone, environmental condition, type explant and species on callus induction of *Crataegus*

Leaf-derived calli of hawthorn grown on MS media supplemented by different concentrations of 2,4-D combined with different concentrations of NAA and KIN began to appear after 1 weeks. Data presented in Tables 1, 2 and 3 demonstrate the effects of using 8 different growth regulators combinations on percentage of calli Induction. Results showed that in ex-vitro leaves in dark conditions treatment 8 produced the maximum percentages of callus (100%). Whereas in the light conditions treatment 5 produced the maximum percentages of callus (21.6%) in species *C. meyeri*. In in-vitro leaves in dark and light conditions treatment 8 produced the maximum percentages of callus (100%) and this leaves species *pseudoheterophylla* produced the maximum percentages of callus (100%) in dark conditions.

Effect of different concentration of hormone and species on Callus Growth

To evaluate the effect of different concentration of hormone and species on Callus Growth, during five weeks after culture, the size of callus [(width + length)/2] (Fig.1,2) and callus fresh/dry weight (Fig. 3,4) on different concentration of hormone and species

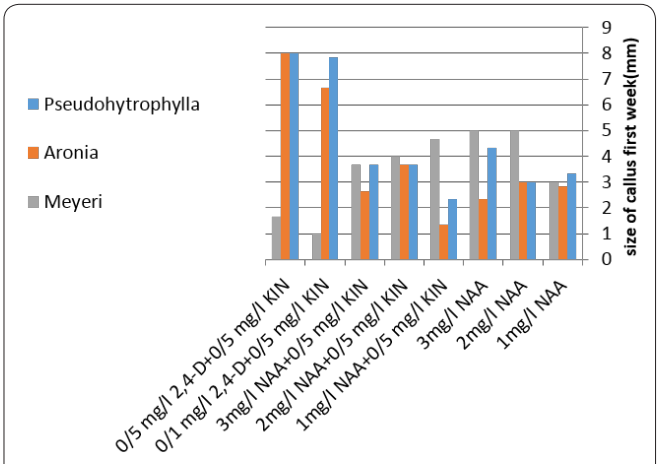

Figure 1. The comparison of hormone compositions on callus size in different species in hawthorn first week after culture.

Table 1. Percentage of callus induction from *in vitro* leaves, ex vitro leaves on MS media under dark and light conditions after four weeks (*C. meyeri*).

Explant	Callus induction in light conditions (%)								Callus induction in dark (%)							
Treatment	T1	T2	T3	T4	T5	T6	T7	T8	T1	T2	T3	T4	T5	T6	T7	T8
In vitro leaves	64.3	69.0	71.0	71.6	76.3	78.6	97.0	100	79.0	81.3	80.0	85.6	88	88.3	100	100
Ex vitro leaves	14.0	12.6	16.3	14.3	21.6	15.0	0.0	0.0	64.6	65.6	80.6	80.0	83	85.0	100	100

Table 2. Percentage of callus induction from *in vitro* leaves, ex vitro leaves on MS media under dark and light conditions after four weeks (*C. pseudoheterophylla*).

Explant	Callus induction in light conditions (%)								Callus induction in dark (%)							
Treatment	T1	T2	T3	T4	T5	T6	T7	T8	T1	T2	T3	T4	T5	T6	T7	T8
In vitro leaves	87.3	91.0	93.0	88.66	93.66	95.33	82.3	100	100	100	100	100	100	100	100	100
Ex vitro leaves	13.3	13.33	13.66	13.33	18.66	16.33	0	0	68.66	66.33	78	76.3	82.3	80.6	75.6	100

Table 3. Percentage of callus induction from *in vitro* leaves, ex vitro leaves on MS media under dark and light conditions after four weeks (*C. aronia*).

Explant	Callus induction in light conditions (%)								Callus induction in dark (%)							
Treatment	T1	T2	T3	T4	T5	T6	T7	T8	T1	T2	T3	T4	T5	T6	T7	T8
In vitro leaves	65.3	68.66	72.0	72.0	75.33	79.66	97.3	100	80.0	82.0	80.0	84.0	88	82.6	100	100
Ex vitro leaves	14.0	14.0	15.33	15.33	21.33	17.66	0	0	65.66	64.33	79.66	77.3	84.6	83.6	100	100

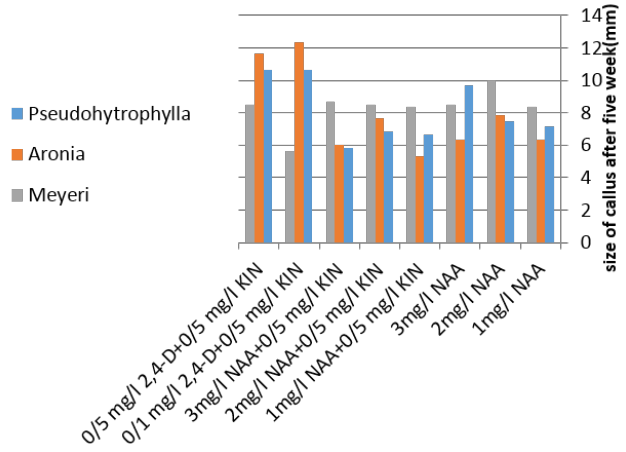

Figure 2. The comparison of hormone compositions on callus size in different species in hawthorn five week after culture.

were recorded. Results showed that treatment type gave statistically significant effect on callus growth (p<0.01). Treatments 7 and 8 supplemented with 0.1 mg/L 2,4-D + 0.5 mg/l KIN and 0.5 mg/L 2,4-D + 0.5 mg/l KIN that were gave the highest mean size of callus (12.33 and 11.66 mm respectively) five week after culture compared to the other treatment. In evaluate the effect of species type on Callus Growth (size of callus), results showed that in the first week species *C. pseudoheterophylla* and *C. aronia* gave the highest mean size of callus (8 mm) compared to the other species. However in the next few weeks were not significant differences between the species. Result showed that the highest mean callus fresh weight in first week (1061.12 and 1059.84 mg) gave in treatment 8 and 7 respectively. While that the five weeks after culture, highest mean callus fresh weight (1093.49 mg) was observed in the treatment 8. The statistical analysis showed that there were significant differences between species for callus growth (p < 0.01). The *C. pseudoheterophylla* species gave the highest mean callus fresh weight (1093.49 mg) compared to the other species after five weeks culture. In evaluate the effect of type species and concentration

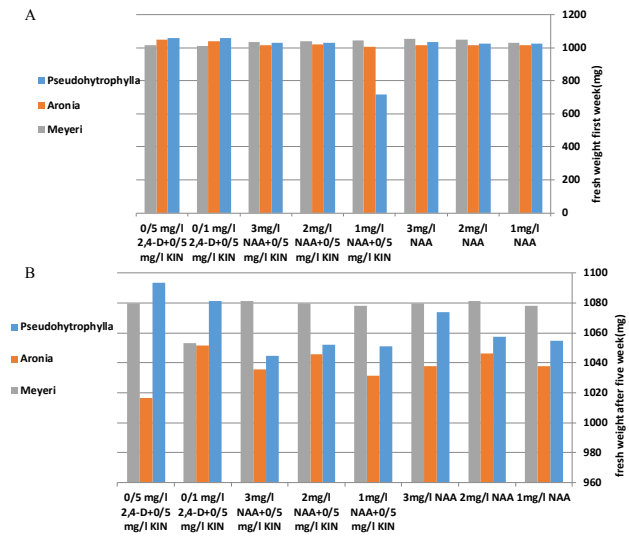

Figure 3. A) The comparison of hormone compositions on fresh weight in different species in hawthorn first week after culture. B) The comparison of hormone compositions on fresh weight in different species in hawthorn five week after culture.

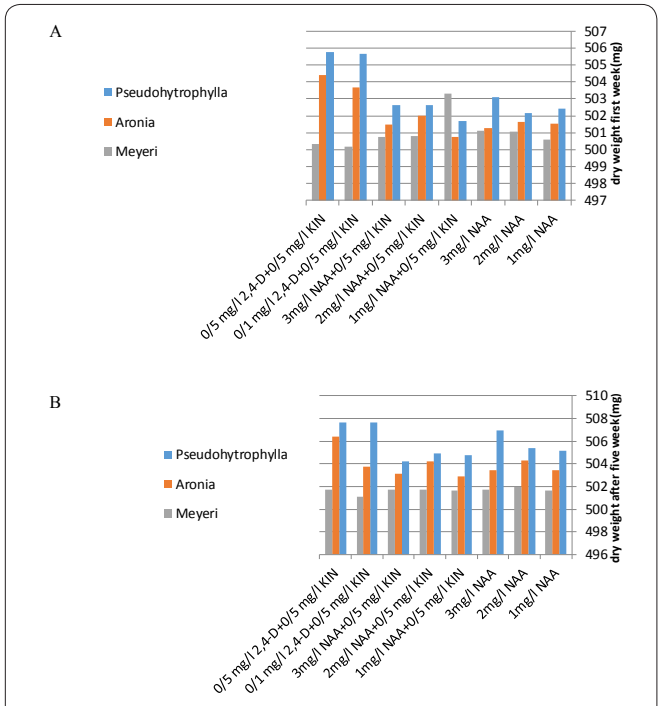

Figure 4. A) The comparison of hormone compositions on dry weight in different species in hawthorn first week after culture. B) The comparison of hormone compositions on dry weight in different species in hawthorn five week after culture.

of hormone on Callus Growth (dry weight of callus) showed that in the first two weeks species *C. pseudoheterophylla* gave the highest mean dry weight of callus (505.76 mg) compared to the other species. In the five weeks after culture the *C. pseudoheterophylla* gave the highest mean dry weight of callus (507.68 mm). Result showed that the highest mean callus dry weight (507.68 mg) gave in treatment 0.1 mg/l 2.4-D + 0.5 mg/l KIN and 0.5 mg/l 2.4-D+0.5 mg/l KIN.

Effect of different concentration of hormone and species on percent and term viability of callus

Significant differences were recorded under different treatment and species (Fig.5). Results showed that treatment type gave statistically significant effect on percent and term viability of callus (p<0.01). The highest mean percent and term viability of callus (100% after six month) gave in treatment 7 and 8 supplemented by 0.1,0.5 mg/L 2,4-D + 0.5 mg/l KIN. Also significant differences were recorded under different species. So the highest mean percent and term viability of callus (100% after six month) gave in *C. pseudoheterophylla* and *C. meyeri* species.

Effect of different concentration of hormone, species, culture medium on somatic embryogenesis

To evaluate the induction of somatic embryos from medium MS and 1/2 MS were used without any hormonal treatment, medium MS and 1/2 MS with concentration1, 2 and 3 mg/l TDZ, medium MS and 1/2 MS with concentration 5 to 2.5 to 0 mg/l 2,4-D and medium MS and 1/2 MS with concentration 2.5 to 0 mg/l 2,4-D (for two weeks) and then transferred to MS and 1/2 MS medium (without any hormonal treatment) until the end of the culture period (three months). The result showed that none of the treatments and species was not show somatic embryogenesis.

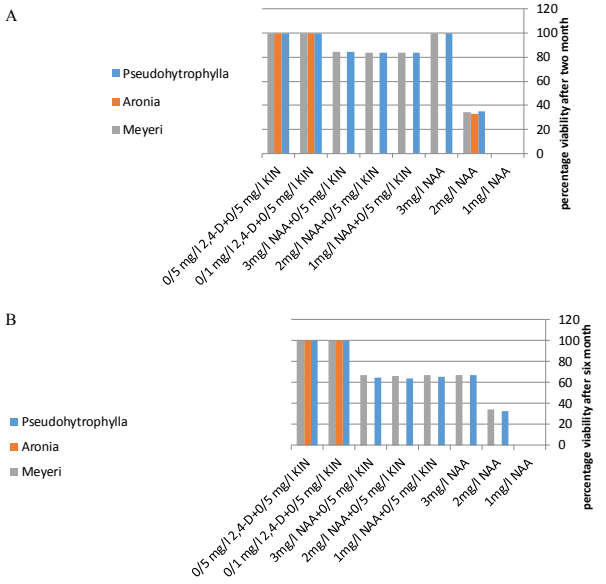

Figure 5. A) The comparison of hormone compositions on percentage viability in different species in hawthorn two month after culture. B) The comparison of hormone compositions on percentage viability in different species in hawthorn six month after culture.

Discussion

Callus is a dedifferentiated and unorganized mass of parenchyma cells, produced as a result of dedifferentiation from differentiated tissues and induced by exogenous factors and growth regulators under *in vitro* conditions (35,36,37,38). Regeneration from callus can be achieved via organogenesis or somatic embryogenesis. Every differentiated plant tissue is totipotent, but the conditions required for dedifferentiation varies from species to species and even tissue to tissue within the same plant (39). Callus cultures are useful in the amplification of limited plant material. In addition, plants regenerated from long term derived callus are also a source of somaclonal variation which results either from an existing genetic variability or induction of mutations as a result of application of plant growth regulators(40). The effect of using different concentrations of 2,4-D and NAA as auxin complemented by KIN as cytokinin on callus induction, callus growth and percent and term viability of callus and embryogenesis in leaf explants of *C. azarolus, C. pseudoheterophylla* and *C. meyeri* were investigated. Our results showed that the best callus induction in in-vitro and ex-vitro leaves as percentage were observed in dark conditions in MS medium supplemented by 0.5 mg/L 2,4-D +0.5 mg/l KIN and 0.1 mg/L 2,4-D +0.5 mg/l KIN. In the between species the maximum percentages of callus in the dark conditions was absorbed in in-vitro leaves s in the *C. pseudoheterophylla* species and ex-vitro leaves was in the *C.aronia* species. Cytokinins are required for callus induction and cell division (41). Callus production depends on the combination hormones are used and balance between the hormones auxin and cytokinin morphogenetic factor is decisive and important (42). Callus weight, color and structure depended on the tomato genotypes and experimental conditions. Callus cultures constitute currently an important source of genetic variability, which is of considerable importance in the breeding of many species of cultivated plants. Their induction depends on numerous factors – different for different cultivars and species (43). Among the important factors it is also the choice of the basic medium and culture conditions (light or darkness). Also, the morphology of the callus can be determined by manipulating the growth regulators. Most often it depends on the auxin to cytokinin ratio in the medium (43, 44,45). The balance between auxin and cytokinin application is needed for callus formation (46). The information regarding the effects of various plant growth regulators as culture medium supplements on Hawthorn tissue culture is lacking. In all our experiments produced more callus when incubation was occurred in the dark. In agreement with our results

Jaramillo and Summers (1991) (47) reported that tomato anthers produced more callus when incubated in the dark, whereas callus exposed to a 16-h photoperiod browned earlier and ceased growth. Light is a very important physical factor for callus induction, cell growth and production of plant secondary metabolites. However, the degree of responsibility to light is depended on cell type, genus and species plant. In glutinous rice cultivar TDK1, seeds cultured under light conditions gave higher average callus formation frequency and larger average size of callus than those cultured under dark conditions (48). Similar results were also observed in the Indica rice cultivar RD6 (49). In Japonica rice cultivars Nipponbare and Kitaake, cultivation of cells under light conditions promoted higher growth than that cultivated under dark condition (50). The differences between our results with those previously reported are lightly due to the difference in cell type , genus and species plant. The type of plant growth regulators and conditions of explants incubation were found to have significant effect on callugenesis and browning of explants.

In agreement with our results, Maharik *et al* (2009) (51) reported that equal levels of cytokinin and auxin were found to induce the best callus formation. In contrast with our results, Alamdari and Safarnejad (2010) (52) reported that Medium MS containing 0.1 mg/l IAA and 1.5 mg/l BA and MS medium containing 4 mg/l TDZ as the most suitable combination of known hormone for callus hawthorn that may be different species, time collecting and age of tree. Our result showed that the highest mean callus fresh weight (1093.49 mg) gave in treatment, which had almost the same proportion of auxin and cytokinin that is in agreement with Assareh et al (2007) (53). In agreement with our results, Chaâbani et al (2015) (54) reported that in *Crataegus azarolus* (hawthorn), the supplementation of 1.0 mg/L 2,4-D and 1.0 mg/L BAP to MS medium was found to be the most efficient for callus induction (as percentage and fresh weigh). Based on our knowledge to date, studies have not been conducted on somatic embryogenesis in hawthorn. Lioyd *et al* (1988) (55) reported that their organogenic cells contained few starch grains. Perhaps this observation could help researchers distinguish cells with organogenic potential from recalcitrant cells. However, regeneration of adventitious shoots or somatic embryos are very rare occurrences in roses (56,57). A significant literature on auxin biosynthesis, metabolism, and transport in embryos that has grown out of extensive analysis of carrot somatic embryos showed that auxin appears to play important roles both in the induction of embryo

development in culture and in the subsequent elaboration of proper morphogenesis in embryo development (58,60,59). The role of exogenous auxin in somatic embryo induction appears to depend on the nature of the explant used in the experiment. For example, petiole explants (61), hypocotyl explants (62), and single cells isolated from established suspension cultures (71). Cytokinin such as KIN and BAP at the start of somatic embryogenesis is useful in a number of wood species used are usually by auxin in the environment. Analysis reports showing that cytokinins during somatic embryogenesis in woody plants is minimal (63). Genotypes, between different species have different capacity embryogenesis, the genetic differences in the capacity of embryogenesis in enabling the key elements included in embryogenesis routes. In addition, the specific genotypes may have the best capacity to regenerate their unique needs (64). Yeung (1995) (65) described the effect of the application of external auxin induction especially 2.4-D of somatic embryogenesis. Assareh et al (2007) (53) found that in *Eucalyptus viminalis* after transferring callus culture medium containing 1mg/l 2.4-D was not produce embryogenic callus. Nugent and Chandler (2001) (25) reported that do not affect 2.4-D and other synthetic auxin in the embryo callus formation in tissue culture. In contrast with our results, Ebadi *et al* (2001) (66) reported that the hormone - free MS suitable medium is a culture for differentiation of somatic embryos. Klimaszewska *et al* (2001) (67) reported that the somatic embryogenesis success to several factors such as species, type of genotype explants medium composition and the amount of time collecting. The age and physiological conditions of the parent culture medium and the right combination of hormones also affect the success of organogenesis cell cultures (68). Absence of pre - embryonic structures can be fine - type genotype or combination of hormones used in the culture medium (69). Callus induction efficiency is influenced by various external factors (70, 71, 72). This plant can be used as a medicinal plant else (73)

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